Investigation of Ecotoxicity of Ionic Liquids Using Different Microbes

by

Nur Hidayah binti Shahrul-Amar

Dissertation submitted in partial fulfillment of
the requirements for the
Bachelor of Engineering (Hons)

(Chemical Engineering)

MAY 2014

Universiti Teknologi PETRONAS
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CERTIFICATION OF APPROVAL

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Approved by,

_____

(Supervisor: Dr Muhammad Moniruzzaman)

(Co-Supervisor: AP Dr Cecilia Devi A/P Wilfred)

UNIVERSITI TEKNOLOGI PETRONAS
TRONOH, PERAK
MAY 2014

CERTIFICATION OF ORIGINALITY

This is to certify that I am responsible for the work submitted in this project, that the original work is my own except as specified in the references and acknowledgements, and that the original work contained herein have not been undertaken or done by unspecified sources or persons.

NUR HIDAYAH BINTI SHAHRUL-AMAR

ABSTRACT

The vast development of a huge number of novel ionic liquid is resulted from the intense growth in research of this interesting subject. The world has taken a deep concern in the development of ionic liquid for the advancement in various industries, including microbiological fields. The use of microorganism as replacement for chemical catalysts in synthetic processes may be further increased by the replacement of conventional organic solvents, with this so called "designer solvent" known as ionic liquids. Ionic liquids have been widely reported as "green" solvent due to their negligible vapour pressure. However, only few reported the toxicity level of ionic liquids when tested against microorganism. This review will discuss matters of the toxicity of different concentration of ionic liquids, namely 1- butyl- 3-methylimidazolium dimethylphosphate (BMIM DMP), 1- methyl- 3-methylimidazolium dimethylphosphate (MMIM DMP), 1-butyl-3-methylimidazolium octyl sulfate (BMIM OSU) and 1- butyl- 3- methylimidazolium hydrogen sulfate (BMIM HSO_4) towards selected microorganisms; *Aeromonas Hydrophila, Eschericia Coli, Listeria Monocytogenes,* and *Staphylococcus Aureus* using Minimum Inhibitory Concentration (MIC) test. MIC test will be evaluated based on the graph obtained for EC50, which is the ionic liquid concentration that gives half maximal response. From this study, we can determine if the toxicity level of ionic liquid is high, the ionic liquid can act as an antimicrobial for the pharmaceutical industry, where else nontoxic ionic liquid can be used for bioprocess/biotechnology industry.

ACKNOWLEDGEMENT

First and foremost, I would like to praise God the Almighty for His guidance. Though difficulties occurred, His guidance and blessing has gave me the chance to go through this challenging project till the end.

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Nomenclatures/Abbreviations

IL(s)	Ionic liquid(s)
EC50	Half Maximal Effective Concentration
MIC	Minimum Inhibitory Concentration
MMIM DMP	1,1-dimethyl imidazolium dimethyl phosphate
BMIM DMP	1-butyl-3-methyl imidazolium dimethyl phosphate
BMIM OSU	1-butyl-3-methyl imidazolium octyl sulphate
BMIM HSO4	1-butyl-3-methyl imidazolium hydrogen sulphate
UTP	Universiti Teknologi PETRONAS
ME	Microemulsion
ACV	Acyclovir
Tween 80	Polyoxyethylene sorbitan monoeleate
Span 20	Sorbitan laurate
IPM	Isoprophyl myristate
API	Active Pharmaceutical Ingredients
ICH	International Conference of Harmonization

CHAPTER 1

INTRODUCTION

1.1 Background of Study

A group of organic salts that combines cation and anion is called ionic liquid (IL). Lower melting point of ILs compared to the normal salts has made it becomes the substitute for "green" solvent (Ghanem et al, n.d.). The characteristics of ILs; lack of vapour pressure, good thermal and chemical stability and very good "separation" in both organic and inorganic solvent make it more favorable compared to conventional solvent (Siodmark, 2012). IL is also known as "designer solvent" as we can design the properties such as polarity, acidity/alkalinity value of the ionic liquids and etc according to the industries requirements.

Although this subject is still considered as new in various industries, it has developed an extensive range of ILs ion, including electrolytes, biomass processing, synthesis, separation, and advanced materials. Figure 1 shows some of the applications of ILs in several industries.

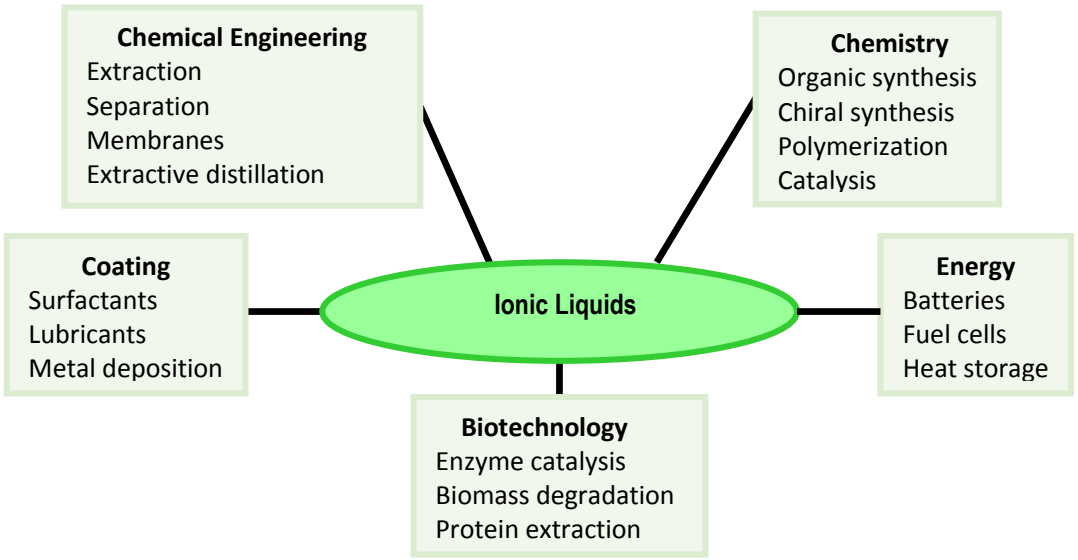

Figure 1 Applications of Ionic Liquids in various industries

Besides all of the functions of ILs stated above, ILs has also being introduced in pharmaceutical industries. Recent findings has stated that IL based microemulsion (ME) as a potential carrier of sparingly soluble drug are getting more attention in this industry. The transdermal drug delivery has soluble or insoluble drugs in the water and most of the organic solvents. In order to overcome the challenges, Moniruzzaman et al. (2010) has stated that IL-in-oil ME were employed to increase the solubility of a sparingly soluble drug to enhance its topical and transdermal delivery.

Nevertheless, it has been reported in article entitled "Toxicity of Ionic Liquids" by Zhao et al (2007) that many commonly used ILs have their certain amount of toxicity. The chemists who specialized working in the area of green chemistry have taken their concern regarding the toxicity in IL. This is due to the "residual solvents" or "organic volatile" that resulted from the reaction media in the final product which has produced contamination (Siodmark et al 2012). Synthesizing IL with the combination of anion and cation together with the alkyl chain, the chemicals have different label of hazardous including corrosive (i.e. 1-methylimidazole), harmful (i.e. sodium dicyanamide) and toxic (i.e. Li[Tf_2N]) so the assumption that all risk hazards of these chemicals will fade away due to their conversion into ILs cannot be confirmed.

According to Pretti et al (2009), the toxicity of IL is strongly affected by the cationic head group as it decreases from aromatic heterocyclic nitrogen, which contains

compounds (pyridinium and imidazolium) to non-aromatic cyclic and acyclic compounds (pyrolidinium, ammonium and morpholinium). Reichert (2005) also stated in his article that interaction of cation and anion of ILs play an essential role in order to determine the properties of the ILs.

Another studies are found that the side chain of ILs affect the toxicity level towards the microbes. The longer the side chain, the IL will become more toxic. This statement is also supported by the Pretti et al (2009) and Cho et al (2007) that longer alkyl chain resulted the increasing of toxicity level.

1.2 Problem Statement

Ionic liquid has been proven to be developed in numerous industries. Despite of its ability to be used for multiple purposes, the toxicity data for 1, 3-dimethylimidazolium dimethylphosphate (MMIM DMP), 1- butyl- 3- methylimidazolium dimethylphosphate (BMIM DMP), 1- butyl- 3- methylimidazolium octyl sulfate (BMIM OSU) and 1- butyl- 3- methylimidazolium hydrogen sulfate (BMIM HSO_4) towards selected microbes are still limited. Thus, the "greenness" of ILs compared to conventional organic solvents are still questionable. This study will investigate the ecotoxicity of ionic liquids towards selected microbes.

1.3 Objectives

The objectives of this research paper are:

- To determine EC50 for ILs; namely MMIM DMP, BMIM DMP, BMIM OSU and BMIM HSO_4 towards selected microorganisms. *(Aeromonas Hydrophilia, Eschericia Coli, Listeria Monocytogenes* and *Staphylococcus Aureus)*
- To study the effect of anion and cation towards the toxicity level of ILs
- To study the effects of toxicity of ILs towards various industries, especially pharmaceutical industry

1.4 Scope of Study

The experiment will be conducted in Toxicity Laboratory of Ionic Liquids Research Centre, Universiti Teknologi PETRONAS (UTP). The results will be evaluated upon ecotoxicity basis using Minimum Inhibitory Concentration (MIC) test towards the selected microorganisms. MIC can be done with various materials and methods. This study will focus on only few types of ILs; MMIM DMP, BMIM DMP, BMIM OSU and BMIM HSO_4 with different concentration. It will be conducted on four types of microorganisms; *Aeromonas Hydrophila*, *Eschericia Coli*, *Listeria Monocytogenes* and *Staphylococcus Aureus*. The time taken to evaluate the result would be about 24 hours, depending on the nature of microorganisms. However, the time would be varied as the concentration of ILs need to be identified before achieving a good result.

CHAPTER 2

LITERATURE REVIEW

2.1 Ionic Liquids (ILs)

2.1.1 What is IL?

IL is basically a salt in liquid state. It is largely made up of ions and short-lived ion pairs. IL usually has a melting point below arbitrary temperature, for example 100°C (Rodrigues et al., 2010). When the salt melts without being decomposed or even vaporized, it will yield an IL. ILs are considered as "designer solvents", which means that all the properties i.e. polarity, density, viscosity, hydrophobicity, hydrogen-bonding capability, thermal stability or toxicity, can be adjusted by varying the structure of the component ions to obtain the desired characteristics (Institut fur Angewandte Synthesechemie Technische Universitat Wien, n.d.). The low melting point is resulted from the chemical composition of room temperature ILs. It contains a large irregular organic cation compared to the inorganic equals of molten salts. Lattice energy, which refers to the energy that would be released if the component ions were brought together from infinity are decreased due to the irregularities thus causing the melting point of ionic medium. However, there are some cases that the anions are relatively huge and lowers down the melting point.

2.1.2 Composition of IL

Donata et al (2004) have stated that there are novel combination of cations and anions that may affect the low melting point of ILs. Some of the most commonly used cations according to sequence are N-alkyl-pyridinium, 1-alkyl-3-methyl imidazolium, tetraalkyl phosphonium and tetraalkyl-ammonium, with the pairing of anions from the most immiscible are $[PF_6^-]$, $[N(SO_2CF_3)_2^-]$, $[BR_1R_2R_3R_4^-]$ to the most water miscible anions $[CH_3CO2^-]$, $[CF_3SO_2^-]$, $[NO_3^-]$, $[Cl^-]$ together with the alkyl chains of ethyl,

butyl, hexyl, octyl and decyl. The summarize chart about the composition of IL is as in the Appendices 4.

By the name of "designer solvents", researchers can select any small anions i.e hexaflourophosphate and tetraflouroborate mixed with the large cations for example 1-hexyl-3-methylimidazolium in order to form an IL. So the IL can be "tailored" according to the requirements and necessity of each industry.

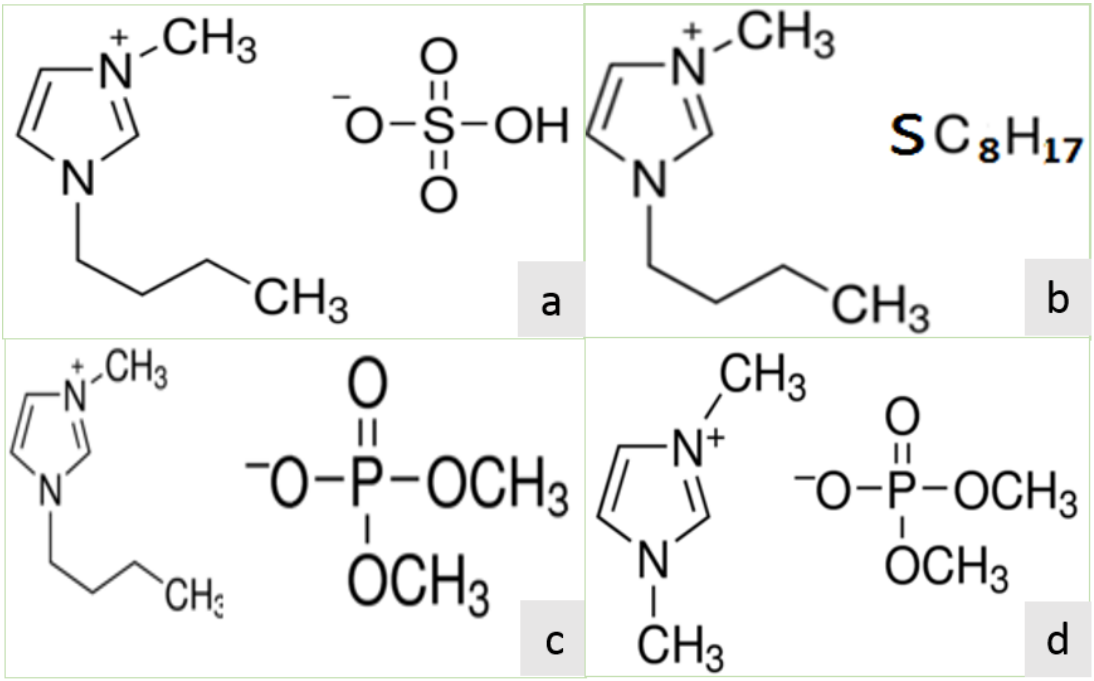

Figure 2 (a) 1- butyl- 3- methylimidazolium hydrogen sulfate (BMIM HSO_4) (b) 1- butyl- 3- methylimidazolium octyl sulphate (BMIM OSU) (c) 1- butyl- 3- methylimidazolium dimethylphosphate (BMIM DMP) (d) 1, 3-dimethylimidazolium dimethylphosphate (MMIM DMP)

2.2 Conventional Salts Vs ILs

Nowadays, the world has more understanding towards the significance of a better planet. All industries are directing their ways to a greener living place. According to Ventura et al (2012), the design of an environmentally and safe solvents are progressively vital in manufacturing process. The IL has been a great founding for a replacement of a conventional organic solvent. The problem with most of the conventional organic solvent are not only hazardous and high toxicity properties, they also costly and waste byproducts from the chemical industries causes environmental problems. Furthermore, prolonged and high concentration exposures

of the organic chemicals can cause occupational diseases (Green Chemistry- Green Engineering, n.d.). Moreover, the conventional salts exhibit a high melting point, i.e. 801°C for sodium chloride and 614°C for lithium chloride, which will minimize their use as solvents in most applications.

On the other hand, IL has been explored for the replacement of conventional organic solvent. The IL may act as solvents and/or (co)solvents and/or reagents in a wide range of pharmaceutical applications due to their "custom made" chemical, physical and biological properties. IL owns properties of having a wide liquid range with melting point around room temperature, good stability in air and moisture, high solubility including inorganic, organic and even polymeric materials. It even has a wide range of solvent polarity and negligible vapor pressure so that makes ionic liquid become low flammability solvent (less toxic) thus minimizing the release of chemical to the environment. Due to the "tunable" characteristics of ILs, there are a very extensive possibility of anion and cations which can be designed with regards to the polarity, hydrophobicity, acidity/alkalinity and etc. (Latala et al, 2005).

Many has agreed and reported that the ILs are "environmental-friendly" and is possible to replace conventional solvents in line for its negligible vapor pressure (Romero et al., 2007). Many has reported that some of the industrial processes have also substituted volatile, polluting hydrocarbon solvents with ILs. Latest studies shows that IL has the potential to react in a fast reaction by pulse radiolysis and the charged species are moving more slowly in ILs compared to the neutral species, which is totally conflicted with the conventional solvents (Wishart, n.d.).

2.3 IL in Pharmaceutical Industry

2.3.1 Developments of ILs in Pharmaceutical Industry

The study of ILs has definitely catch the attention of drug designers and researchers on developing the new findings in medical treatment and also their delivery options. Transdermal drug delivery is one of the options in routing of administration wherein active ingredients were delivered across skin for systematic distribution (Moniruzzaman et al, 2010).

Solubility is very important in designing drugs. Solubility may be defined as the maximum concentration of a substance that may be completely dissolved in a given solvent at a given temperature and pressure. The drugs need to be soluble with a suitable solvent. One way to overcome the problem in poor solubility is to mix with excipients i.e. surfactant. The purpose of adding up excipient is to bulking up formulations that contain potent active ingredients. Table of solubility of a substance is given in the Appendices 3.

Moniruzzaman et al (2010) has found that IL in oil microemulsion (ME) were engaged so that the solubility of sparingly soluble drug will be increased. A mixed composite between nonionic surfactants; polyoxyethylene sorbitan monooleate (Tween 80) and sorbitan laurate (Span 20), which can lower down the surface tension between two liquids or between solid and liquid together with isopropyl myristate (IPM) as an oil phase, and IL; MMIM DMP as pseudophase. Midst of all the ratios that has been experimented in synthesizing ME, acyclovir (ACV); which has been taken as a model of a sparingly soluble drug showed a great solubility and skin permeation from the formation of 3:2 of Tween 80 and Span 20. It has been shown that higher Tween 80 to Span 20 that is above the ratio of 1:1 will reduce the solubility of ACV in formulations. This is due to the formation of stable ME droplets with a large interface compared to the other ME.

Siodmiak et. al. (2012) has stated that synthesis of pharmaceutical compounds are responsible for organic contamination of the final product which referred as “residual solvents” or “organic volatile impurities”. International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use and pharmacopoeias have set the acceptable limits contaminants in process of manufacturing drugs. There are certain guidelines to distinguish residual solvents in drug substances; which are (a) solvents to be avoided (b) solvents to be limited (c) solvents with low toxic potential and (d) solvents without adequate toxicological data. Exposures to even low levels of the solvents with such impurities present in the active pharmaceutical ingredients (API) may result genetic mutations and cancer.

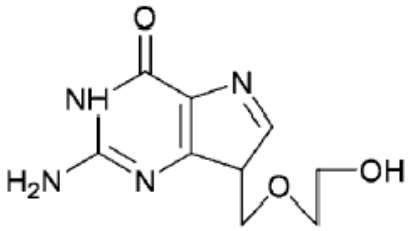

Figure 3 Acyclovir (ACV)

2. 3.2 Microemulsion (ME) System

Moniruzzaman et al (2010) has found that IL can assist in the process of delivering drugs especially for the sparingly soluble or insoluble drugs in water and most organic liquids. A non-aqueous ME has been developed consists of IL; MMIM DMP and two nontoxic surfactants composites; Tween 80 and Span 20. The function of surfactant are to lower down the surface tension of liquids or the tension between a solid and liquid. They prevent the accumulation of ionic liquid with the drug.

Danielsson and Lindman (1981) have introduced a definition of ME as "A system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution". There are three basic types of ME; direct (oil dispersed in water, o/w) comprise water as the continuous medium, reversed (water dispersed in oil, w/o) comprise oil as the continuous and bicontinuous which has almost equal amounts of water and oil, depending on the relative ratios of the constituting components.

According to Queen's University (2010), ME is basically prepared by oil mixing with an aqueous phase with the help of dispersion agent or what we called as surfactant. It is sometimes also added with a cosurfactant, which is generally an alcohol of an intermediate chain length. Some differences between emulsion and ME are:

- ME droplets are obviously smaller than usual emulsion, which is at least about one order of smaller magnitude, 10-100 nm.
- ME form spontaneously compared to course emulsion which require vigorous stirring
- ME are more stable with respect to separation into their components, meanwhile emulsion have a degree of kinetic stability but separate ultimately

With all these differences, ME is more suitable to be used for sparingly soluble drug molecules as a drug carrier. ME is essential in this study because the necessity to study and measure the toxicity of IL in bulk size. The IL alone cannot be used as they are highly hydrophilic, which means that it has tendency to dissolve in or mix with water. The ME system, comprises of water, oil and amphiphiles have been found to be the best solution in drug delivery due to its size, stability, biocompatibility and straightforward preparation (Moniruzzaman et al, 2010).

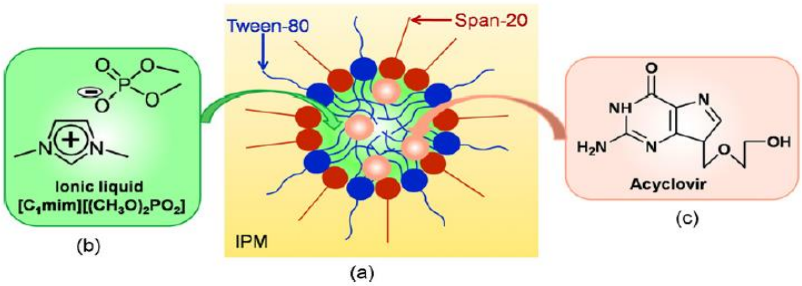

Figure 4 (a) Schematic representation of ionic liquid-in-oil (IL/o) ME containing drug molecules. Chemical structure of IL (b) and ACV (c)

2.3.3 Role of Surfactants in Formulation of ME Systems

$$\Delta G_f = \gamma \Delta A - T\Delta S$$

Where

ΔG_f is the free energy of ME formation

γ is the interfacial tension at oil-water interface

ΔA is the change in interfacial area (associate with reducing droplet size)

T is the absolute temperature

S is the system entropy

According to Alany et al (n.d.), above equations shows the proposed simplified thermodynamic model to explain the formation of an ME system. In forming ME, higher entropy ΔS is needed in order for the free energy to deliver. It is a process promoted by entropy due to the increased randomness related with the dispersion of one of two immiscible phases as small droplets in the second phase. The migration of

surfactant molecules to the interface of the two immiscible phases will lower down the interfacial tension. By adding the second surfactant, the interfacial tension can be further reduced which results thermodynamically stability of ME.

The other factor that contributes to ME formation process is the reduction of droplet size, which will resulted an increase of ΔA as the surface area is increased. Relatively high amphiphile concentrations will yield a reduced value in γ, thus gives a negative value for ΔG_f and eventually forming a ME.

2.4 Introduction and Usage of Microorganism

Microorganism is a microscopic organism which may be present in a single or multicellular organism. Microorganism is an important element to be taken care of as they are in the Earth's elements cycles; i.e. carbon cycle and nitrogen cycle. Microorganism also act as the recycler for other dead remaining organism or even the waste products. It is used as a replacement for chemical catalyst in synthesis processes. Findings shown a process called "biotransformations", which explains that microorganism can modify a certain compounds by simple chemical well defined reactions. It can be further catalyzed by enzymes (Vasic-Racki, n.d.). Microorganism also being used in the processing plant to ensure the safety and quality in Quality Checking factor (FOSS, n.d). The purpose of testing is to give confidence to the customer towards quality and safety of the products.

Mining industry is an industry that will discharge some recyclable metals; palladium, platinum and rhodium which can pollute the environment, specifically soil and water. Recent findings found that microorganisms and a little amount of hydrogen can be used for the metal recovery (Gauthier et al, 2010). By doing this, the cost of the process has reduced tremendously and it is clearly more efficient than the conventional method. The result is very surprising as microorganism can eliminate almost 100 percent of the palladium from the polluted water.

2.5 Toxicity of IL

2.5.1 Toxicological Research of ILs from Effect of Alkyl Length and Alkyl Groups

Zhao et al (2007) have quoted Stepnowski et al. in studying the acute toxicity of 3-diakylimidazolium (1-ethyl-2-methylimidozalium [C_2mim], [C_4mim], 1-benzyl-3-methylimidazolium) based IL of $[BF_4]^-$ anion. The purpose of the test is to evaluate the toxicity level towards marine ecosystem by using Baltic algae (i.e. Oocystis submarina and Cyclotella meneghiniama). They focused on two things; the effect of alkyl length (C_2 vs C_6) and types of alkyl group (aliphatic vs aromatic) attached to the imidazolium cation. It shows that different algae resulted a different response to IL. For example, Oocystis appeared that it has been "adjusted" to lower concentration of IL, establishing the growing ability has been recovered after 5 days of exposure. He also quoted from Bernot et al (2005) that the acute and chronic toxicity of imidazolium cation based ILs for the purpose of evaluating the effects of toxicants on reproduction and survival of *daphnia magma*. An indicator (median lethal concentration (LC_{50})) was used for the test. As for the outcome, it was found that toxicity of imidazolium-based IL is corresponding to the commonly used solvents in the chemical industry (i.e. ammonia and phenol).

In a nutshell, they established that a shorter alkyl chain (C_1-C_4) gives a lower toxicity level to algae and invertebrates.

2.5.2 Toxicological Research of Ionic Liquids in Microorganism

Zhao et al (2007) has quoted Docherty et al were using the Microtox method to determine the toxicity level of imidazolium and pyridinium ILs to *Vibrio fischeri,* which is a species of bioluminescent bacterium. *Vibrio fischeri* are found within the marine animals for example at the squid bobtail. Free living *Vibro fischeri* survived by living on a decaying organic matter. They report that the longer alkyl chain length on the IL cation leads to a higher level of toxicity. It can be said that when comparing octyl- and hexyl- substituted ILs are more toxic than commonly used industrial organic solvents such as phenol, toluene and benzene.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Project Flowchart

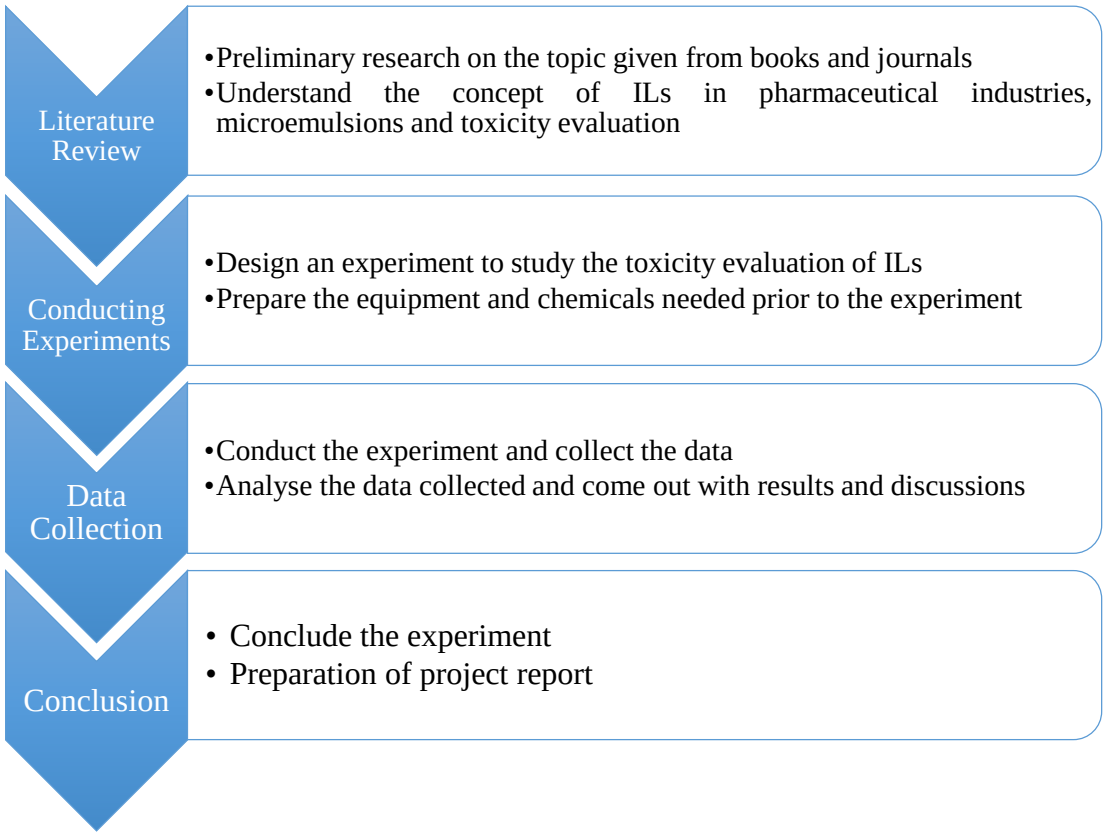

Figure 5 Project Flowchart

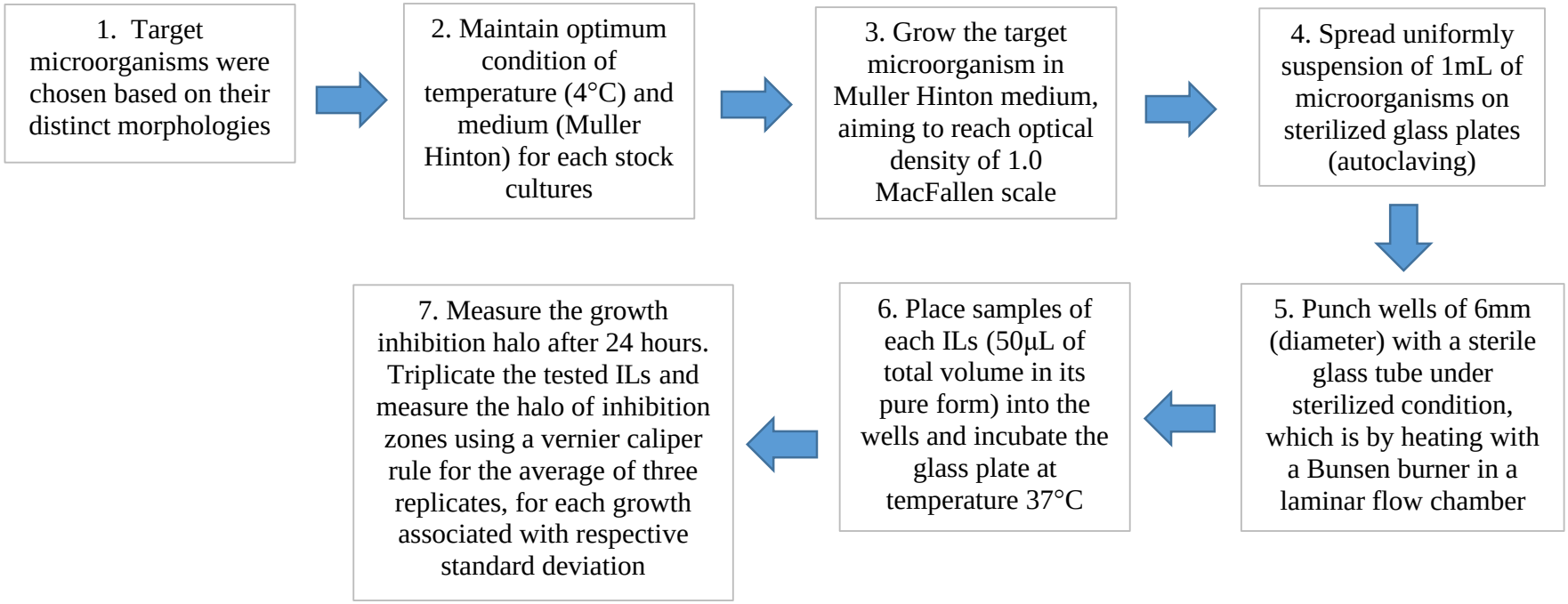

Figure 6 Flowchart for methods of sub culturing microorganism (Ventura, S.P. M. et al, 2012)

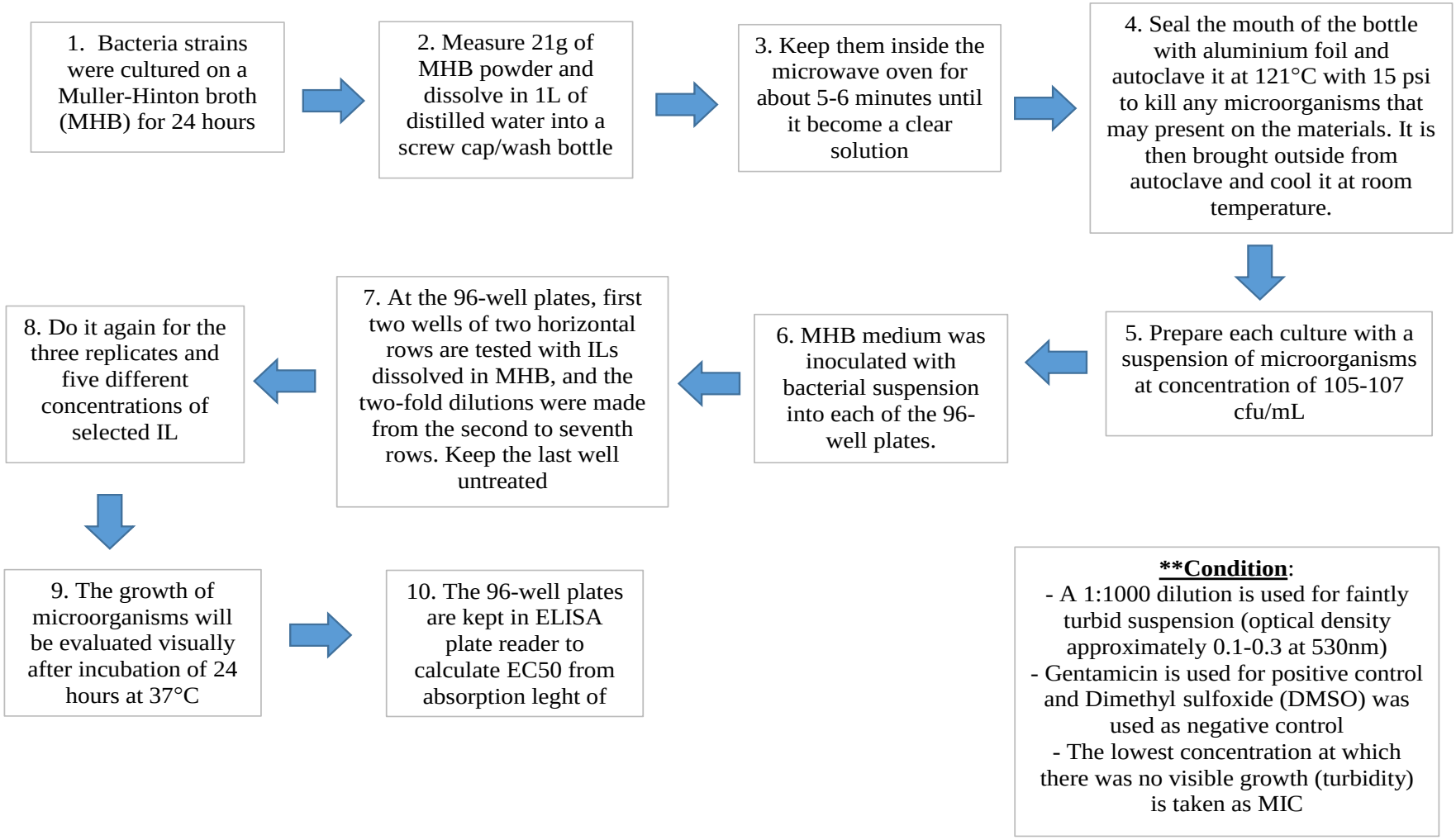

Figure 7 Flowchart in conducting Minimum Inhibitory Concentration (MIC)

3.2 Key Milestones

Table 1 Key Milestone

Activities	Time
Project work continues from previous progress	Week 1- Week 7
Submission of progress report	Week 8
Project work continues	Week 8 – Week 12
Pre-EDX	Week 11
Submission of draft report	Week 12
Submission of Dissertation (soft bound)	Week 13
Submission of Technical paper	Week 13
Oral presentation	Week 14
Submission of Project Dissertation (hard bound)	Week 15

3.3 Gantt Chart

Table 2 Gantt Chart

	Detailed Activity	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	Discuss with Supervisor regarding progress and planning of experiment															
2	Planning for further experiment															
3	Preparation of ionic liquid and microbes															
4	Preparation material for experiment work															
5	Project work continues															
6	Pre EDX															
7	Submission draft report															
8	Submission dissertation (soft bound)															
9	Submission technical paper															
10	Oral presentation															
11	Submission dissertation (hard bound)															

3.4 Experiment Setup and Equipment/Tools Used

3.4.1 Serial Dilution

Serial dilution is a method that is used for identifying the viability of microorganism in an amount of liquid, in another words to determine the MIC of antimicrobial agents. The process is being done by mix the IL with broth in the 96 well plate. As the cell goes to G, the dilution has also decreased to half from the cell before it. The plate filled with ILs, broth and different microorganism as demonstrated in Figure 17.

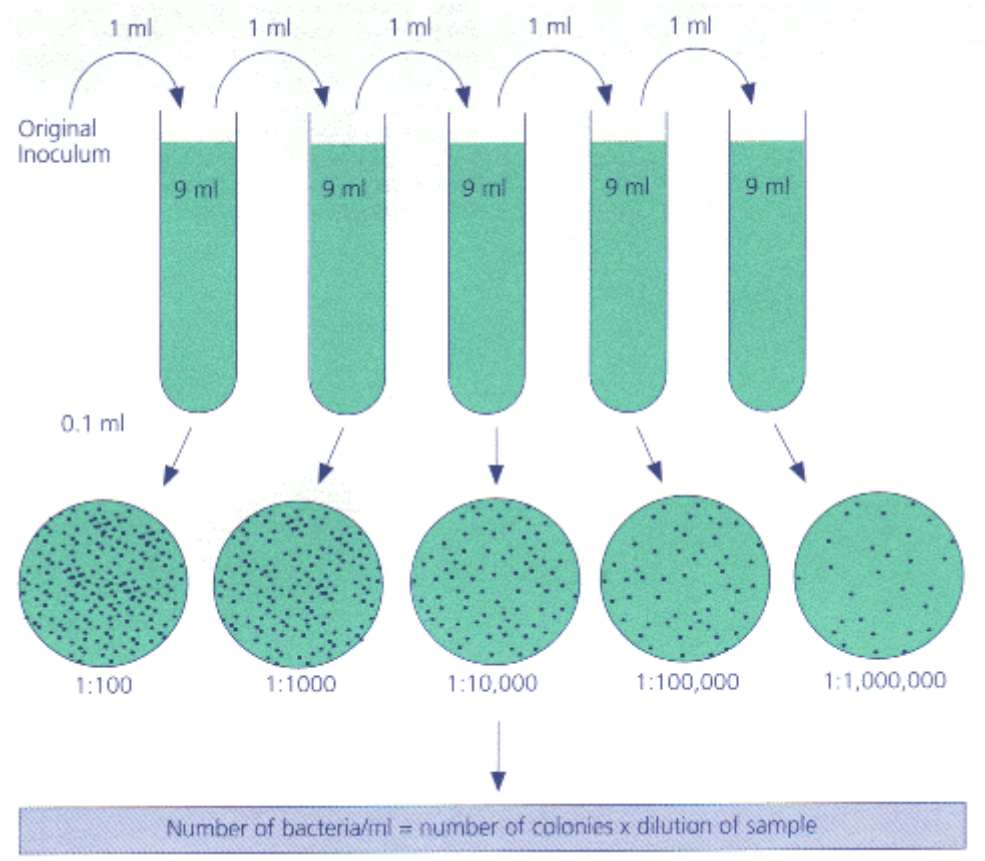

Figure 8 Serial dilution for IL in 96 well plate

3.4.2 Minimum inhibitory concentration (MIC)

MIC test is used to determine the lowest concentration of ILs that inhibits the growth of microorganism; in another word it is to determine Half Maximal Effective Concentration (EC50) for each IL towards the microorganism. EC50 refers to the concentration of a compound where 50% of its maximal effect is observed after a specified exposure duration. It is important in order to identify in which level of

concentration of IL will be toxic towards the selected microorganisms. The test will be conducted after subculture of microorganisms are done. MIC is done by inoculating the organism into a series of wells, which contain broth and serial dilution of selected ILs. After it is incubated for 24 hours, the plate will be analyzed for the bacteria growth. These are some other apparatus/equipment that are being conducted/used throughout the procedure:

3.4.3 Plate reader

Figure 9 Plate Reader

Microplate reader is used for analysis in laboratory. It is designed to detect biological chemical or physical events of samples in microtiter plates. In this case, plate reader is used to analyze the sample reaction of different types of ILs with different types of microorganisms. In this experiment, it analyzes 96 well (8 by 12 matrix) with volume of 200 microliter per well.

3.4.4 Thermo Scientific™ SkanIt ™ Software

Software that is being used to analyze the EC50 for MIC test. It is being measured using several wavelengths. The data is then being transported to Microsoft Excel and graphs are constructed based on the data.

3.4.5 Autoclave

An equipment which is used to sterilize equipment and apparatus by provide a very high pressure saturated steam at 121°C for about 15 minutes, depends on the size of

the equipment and apparatus. It is being used to avoid any bacteria contaminate the equipment/ apparatus that might affect the viability of the microbes.

Figure 10 Autoclave

3.4.6 Optical density for McFarland Standards

This standard is used for setting down the turbidity of bacterial suspension so that the bacteria will be within the approximate extensity as McFarland Standard. It is adjusted by visually comparing the turbidity with McFarland Standards using Wickerham Card. The card is placed behind both tubes of tested microbes and Mcfarland Standard, provided in the presence of good lighting. If the suspension is too dense, the concentration of tested microbe should be diluted using Mueller Hinton Broth. Before further testing, vortex the tested microbe and McFarland standard very well. In other case, if the tested microbe is too dilute, inoculate it with more microbe until it reaches the required turbidity as McFarland standard. There are few standards with different concentration of bacteria that is available to compare. In this case, the experiment is required to use 0.5 concentration of bacteria, which represents 1.5×10^8 bacteria/ml. Refer appendices 5 for different standard number for McFarland standard.

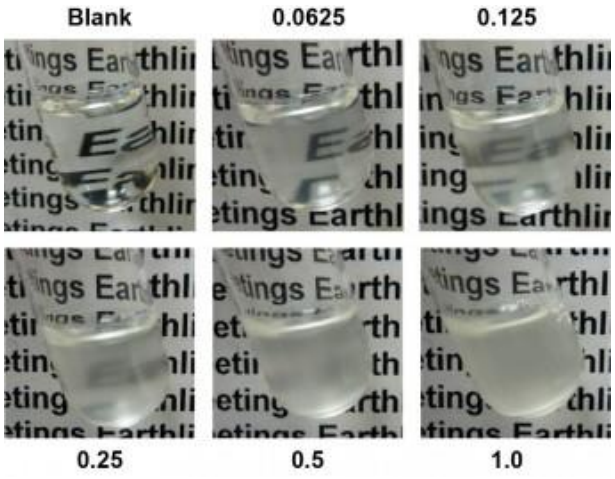

Figure 11 Different Standard number of McFarland Standard

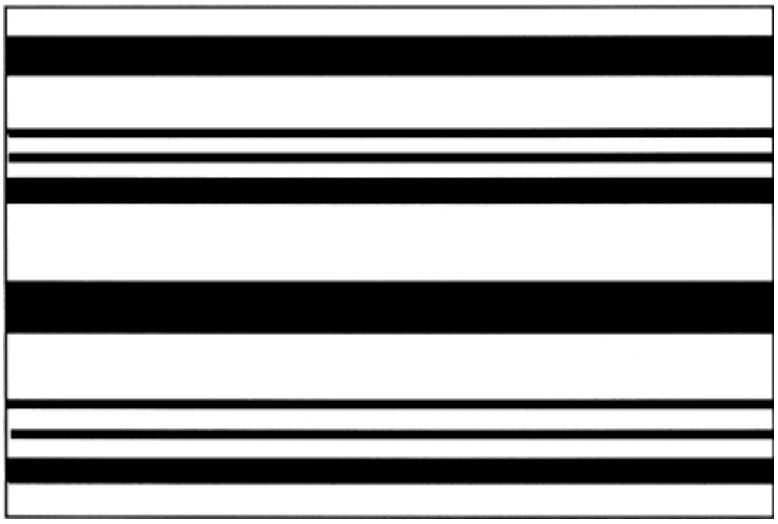

Figure 12 Wickerham Card (Wickerham, L., J. (1951). Taxonomy of yeasts)

CHAPTER 4

RESULT & DISCUSSION

4.1 Results

4.1.1 1, 1-dimethylimidazolium dimethylphosphate (MMIM DMP)

Table 3 EC50 for MMIM DMP in 96 well plate

Value	1	2	3	4	5	6	7	8	9	10	11	12	Concentration
A	0.4204	0.4140	0.4538	0.0598	0.0554	0.0501	0.4562	0.4569	0.4558	0.4522	0.4552	0.5271	25000
B	0.6941	0.7234	0.7474	0.0686	0.0669	0.0655	0.7832	0.7563	0.7714	0.7322	0.7183	0.7597	12500
C	0.8320	0.8345	0.8261	0.0811	0.6445	0.6066	0.8941	0.8339	0.8440	0.8233	0.8292	0.8626	6250
D	0.8436	0.8503	0.8525	0.1960	0.1834	0.4770	0.9488	0.8590	0.8741	0.8500	0.8692	0.9172	3125
E	0.9286	0.8864	0.8931	0.3395	0.3395	0.5244	0.9809	0.9005	0.9357	0.8924	0.8929	0.9352	1562.5
F	0.9494	0.8888	0.8827	0.4521	0.6679	0.4521	0.9839	0.9145	0.9193	0.8824	0.8927	0.9350	781.25
G	0.9878	0.9189	0.9110	0.4828	0.6494	0.4852	0.9469	0.9187	0.9423	0.9158	0.9225	0.9674	390.625
H	1.0229	0.9900	0.9543	0.5442	0.7735	0.5228	1.0274	1.0002	0.9906	0.9520	0.9333	0.9832	195.3125

Table 4 Average EC50 MMIM DMP and Viability for each Microorganism (from left Aeromonas Hydrophilia, Eschericia Coli, Listeria Monocytogenes and Staphylococcus Aureus)

	Average	Viability	Average	Viability	Average	Viability	Average	Viability
A	0.4294	43.41467	0.0551	8.981255	0.4563	45.35485	0.4782	50.00872
B	0.7216	72.96104	0.0670	10.92095	0.7703	76.5655	0.7367	77.05072
C	0.8309	84.00512	0.4441	72.3825	0.8573	85.21635	0.8384	87.67997
D	0.8488	85.81828	0.2855	46.53083	0.8940	88.8576	0.8788	91.90866
E	0.9027	91.26786	0.4011	65.38441	0.9390	93.33709	0.9068	94.84051
F	0.9070	91.69925	0.5240	85.41701	0.9392	93.35697	0.9034	94.47795
G	0.9392	94.96158	0.5391	87.87829	0.9360	93.03227	0.9352	97.8107
H	0.9891	100	0.6135	100	1.0061	100	0.9562	100

Table 5 Summary viability for different concentration of MMIM DMP for each microorganism

	MMIM DMP								Concentration (mg/L)
	Aeromonas Hydrophilia		Eschericia Coli		Listeria Monocytogenes		Staphylococcus Aureus		
	Average	Viability	Average	Viability	Average	Viability	Average	Viability	
A	0.4294	43.41467	0.0551	8.981255	0.4563	45.35485	0.478167	50.00872	25000
B	0.721633	72.96104	0.067	10.92095	0.7703	76.5655	0.736733	77.05072	12500
C	0.830867	84.00512	0.444067	72.3825	0.857333	85.21635	0.838367	87.67997	6250
D	0.8488	85.81828	0.285467	46.53083	0.893967	88.8576	0.8788	91.90866	3125
E	0.9027	91.26786	0.401133	65.38441	0.939033	93.33709	0.906833	94.84051	1563
F	0.906967	91.69925	0.524033	85.41701	0.939233	93.35697	0.903367	94.47795	781.3
G	0.939233	94.96158	0.539133	87.87829	0.935967	93.03227	0.935233	97.8107	390.6
H	0.989067	100	0.6135	100	1.006067	100	0.956167	100	195.3

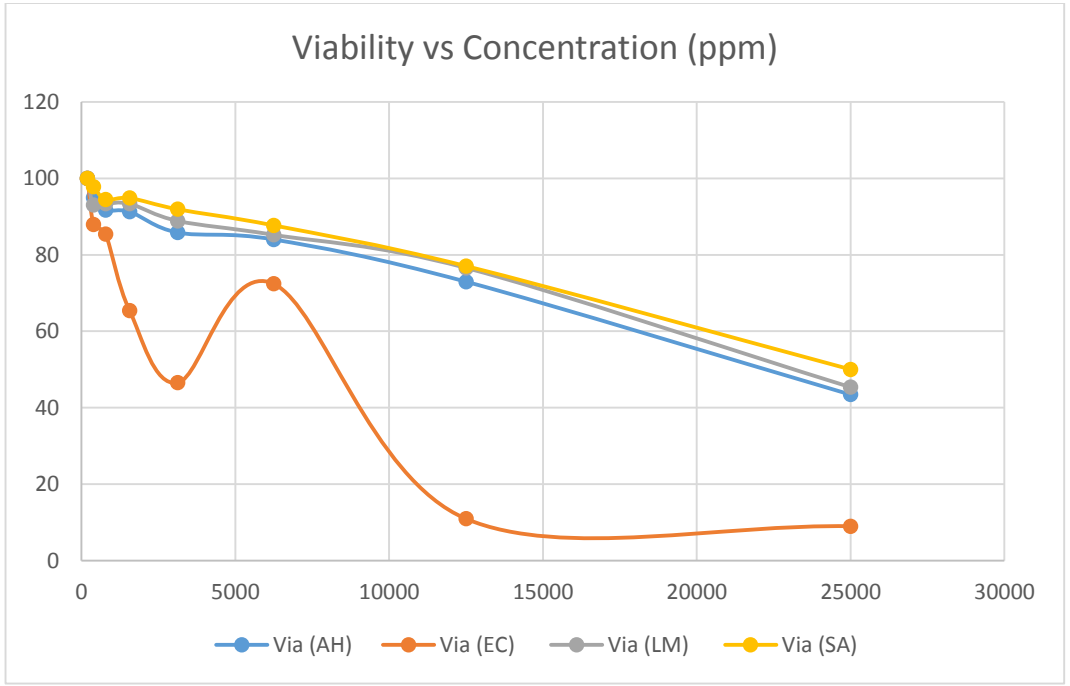

Figure 13 Graph of MMIM DMP viability vs concentration

4.1.2 1-butyl-3-methylimidazolium dimethylphosphate (BMIM DMP)

Table 6 EC50 for BMIM DMP in 96 well plate

Value	1	2	3	4	5	6	7	8	9	10	11	12	Concentration
A	0.3437	0.3379	0.3250	0.0494	0.0499	0.0496	0.2676	0.2298	0.2310	0.2927	0.3014	0.3560	25000
B	0.6120	0.8750	0.7546	0.2637	0.6268	0.1872	0.7452	0.6894	0.7292	0.6953	0.6124	0.5953	12500
C	0.7935	0.7954	0.9885	0.3729	0.4020	0.8517	0.9061	0.9074	0.9967	0.9193	0.9508	0.7548	6250
D	0.8438	1.0040	1.0187	0.6948	0.4314	0.4611	0.9526	0.9890	1.0184	1.0389	1.0609	0.8155	3125
E	0.9150	1.0822	1.0104	0.5082	0.6738	0.3973	1.0683	1.0075	1.0017	1.0399	1.0380	0.8762	1562.5
F	0.9757	0.9079	0.9290	0.5859	0.5519	0.6170	1.0999	1.0642	1.0788	1.0341	1.0345	0.9340	781.25
G	1.0363	1.0807	1.0878	0.6183	0.6002	0.6358	1.0805	1.0656	1.0382	1.1167	1.1213	0.9819	390.625
H	1.1206	1.0526	1.0478	0.5636	0.5537	0.5658	1.0223	1.0193	1.0359	1.0196	1.0179	1.0471	195.3125

Table 7 Average EC50 BMIM DMP and Viability for each Microorganism (from left Aeromonas Hydrophilia, Eschericia Coli, Listeria Monocytogenes and Staphylococcus Aureus)

	Average	**Viability**	**Average**	**Viability**	**Average**	**Viability**	**Average**	**Viability**
A	0.3355	31.25116	0.0496	8.846771	0.2428	23.66856	0.3167	30.8014
B	0.7472	69.59329	0.3592	64.03066	0.7213	70.31032	0.6343	61.69357
C	0.8591	80.01863	0.5422	96.6431	0.9367	91.31438	0.8750	85.09693
D	0.9555	88.9941	0.5291	94.30812	0.9867	96.18197	0.9718	94.51144
E	1.0025	93.37473	0.5264	93.83281	1.0258	100	0.9847	95.76931
F	0.9375	87.32071	0.5849	104.26	1.0810	105.3745	1.0009	97.34163
G	1.0683	99.49705	0.6181	110.1717	1.0614	103.4703	1.0733	104.3863
H	1.0737	100	0.5610	100	1.0258	100	1.0282	100

Table 8 Summary viability for different concentration of BMIM DMP for each microorganism

	BMIM DMP								**Concentration (mg/L)**
	Aeromonas Hydrophilia		**Eschericia Coli**		**Listeria Monocytogenes**		**Staphylococcus Aureus**		
	Average	**Viability**	**Average**	**Viability**	**Average**	**Viability**	**Average**	**Viability**	
A	0.3355333	31.251164	0.0496333	8.8467708	0.2428	23.668562	0.3167	30.801401	25000
B	0.7472	69.593294	0.3592333	64.030658	0.7212667	70.310317	0.6343333	61.693575	12500
C	0.8591333	80.018628	0.5422	96.643099	0.9367333	91.314379	0.8749667	85.096933	6250
D	0.9555	88.994101	0.5291	94.308122	0.9866667	96.181966	0.9717667	94.511444	3125
E	1.0025333	93.374728	0.5264333	93.832809	1.0258333	100	0.9847	95.769306	1562.5
F	0.9375333	87.320708	0.5849333	104.26	1.0809667	105.37449	1.0008667	97.341633	781.25
G	1.0682667	99.497051	0.6181	110.17171	1.0614333	103.47035	1.0733	104.38631	390.625
H	1.0736667	100	0.5610333	100	1.0258333	100	1.0282	100	195.3125

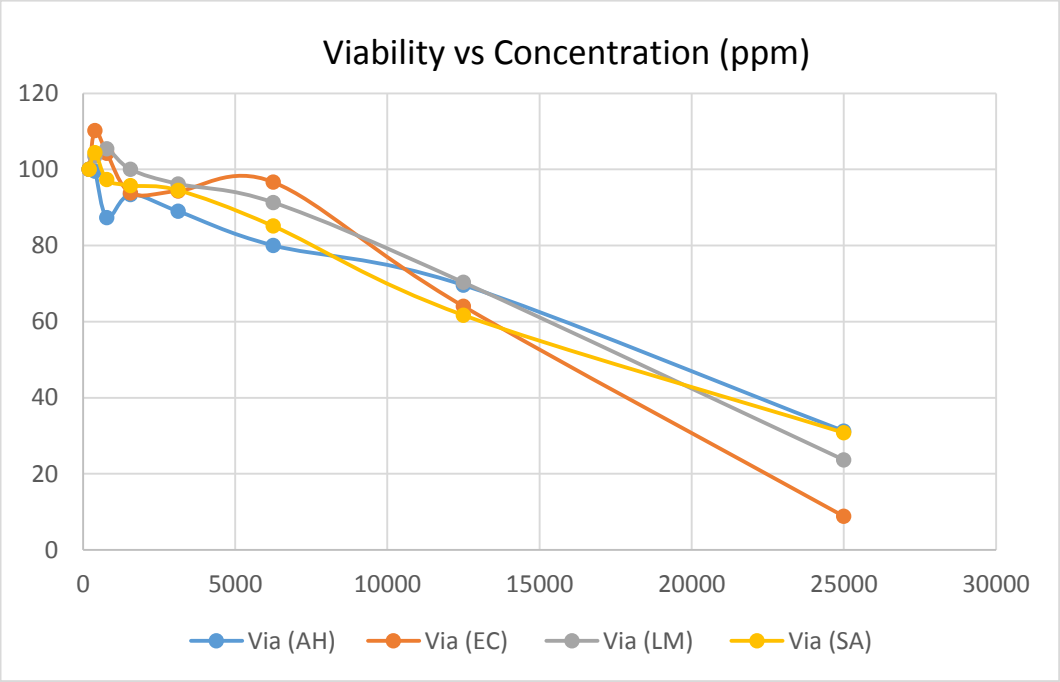

Figure 14 Graph of BMIM DMP viability vs concentration

4.1.3 1-butyl-3-methylimidazolium Octyl Sulphate (BMIM OSU)

Table 9 EC50 for BMIM OSU in 96 well plate

Value	1	2	3	4	5	6	7	8	9	10	11	12	Concentration
A	0.3296	0.3346	0.2912	0.0854	0.0732	0.0746	0.3396	0.3280	0.3464	0.3126	0.3222	0.3467	2500
B	0.6044	0.6309	0.6143	0.3245	0.1262	0.1256	0.6201	0.6294	0.6334	0.6237	0.6219	0.6088	1250
C	0.7295	0.7625	0.7321	0.2493	0.2583	0.2637	0.7409	0.7389	0.7493	0.7259	0.7190	0.7762	625
D	0.8728	0.8447	0.7836	0.3794	0.3698	0.6133	0.8122	0.8066	0.8274	0.7753	0.7943	0.8486	312.5
E	0.9694	0.9104	0.8379	0.4380	0.6864	0.4331	0.8633	0.8919	0.8919	0.8455	0.8480	0.8875	156.25
F	0.9745	0.9360	0.8602	0.4680	0.4583	0.4670	0.9116	0.9305	0.9397	0.8766	0.8933	0.9290	78.125
G	0.9948	0.9529	0.8692	0.4943	0.4961	0.4882	0.9258	0.9229	0.9683	0.9043	0.9170	0.9441	39.0625
H	0.9626	0.9258	0.8808	0.5623	0.5444	0.5459	0.9959	0.9958	1.0007	0.9659	0.9747	0.9929	19.53125

Table 10 Average EC50 BMIM OSU and Viability for each Microorganism (from left Aeromonas Hydrophilia, Eschericia Coli, Listeria Monocytogenes and Staphylococcus Aureus)

	Average	**Viability**	**Average**	**Viability**	**Average**	**Viability**	**Average**	**Viability**
A	0.3185	34.5009	0.0777	14.1111	0.3380	33.886	0.3272	33.458
B	0.6165	66.7919	0.1921	34.8723	0.6276	62.923	0.6181	63.215
C	0.7414	80.3156	0.2571	46.6719	0.7430	74.492	0.7404	75.715
D	0.8337	90.3185	0.4542	82.4458	0.8154	81.747	0.8061	82.434
E	0.9059	98.1403	0.5192	94.2454	0.8824	88.461	0.8603	87.984
F	0.9236	100.054	0.4644	84.3096	0.9273	92.962	0.8996	92.003
G	0.9390	101.723	0.4929	89.4711	0.9390	94.138	0.9218	94.27
H	0.9231	100	0.5509	100	0.9975	100	0.9778	100

Table 11 Summary viability for different concentration of BMIM OSU for each microorganism

	BMIM OSU								Concentration (mg/L)
	Aeromonas Hydrophilia		Eschericia Coli		Listeria Monocytogenes		Staphylococcus Aureus		
	Average	Viability	Average	Viability	Average	Viability	Average	Viability	
A	0.3184667	34.500939	0.0777333	14.111098	0.338	33.885844	0.3271667	33.458326	2500
B	0.6165333	66.791853	0.1921	34.872322	0.6276333	62.922738	0.6181333	63.21459	1250
C	0.7413667	80.315615	0.2571	46.671911	0.7430333	74.492047	0.7403667	75.715016	625
D	0.8337	90.318504	0.4541667	82.445843	0.8154	81.747093	0.8060667	82.433953	312.5
E	0.9059	98.140257	0.5191667	94.245431	0.8823667	88.460767	0.8603333	87.983637	156.25
F	0.9235667	100.05417	0.4644333	84.309573	0.9272667	92.962171	0.8996333	92.002727	78.125
G	0.9389667	101.72252	0.4928667	89.471136	0.939	94.138484	0.9218	94.269644	39.0625
H	0.9230667	100	0.5508667	100	0.9974667	100	0.9778333	100	19.53125

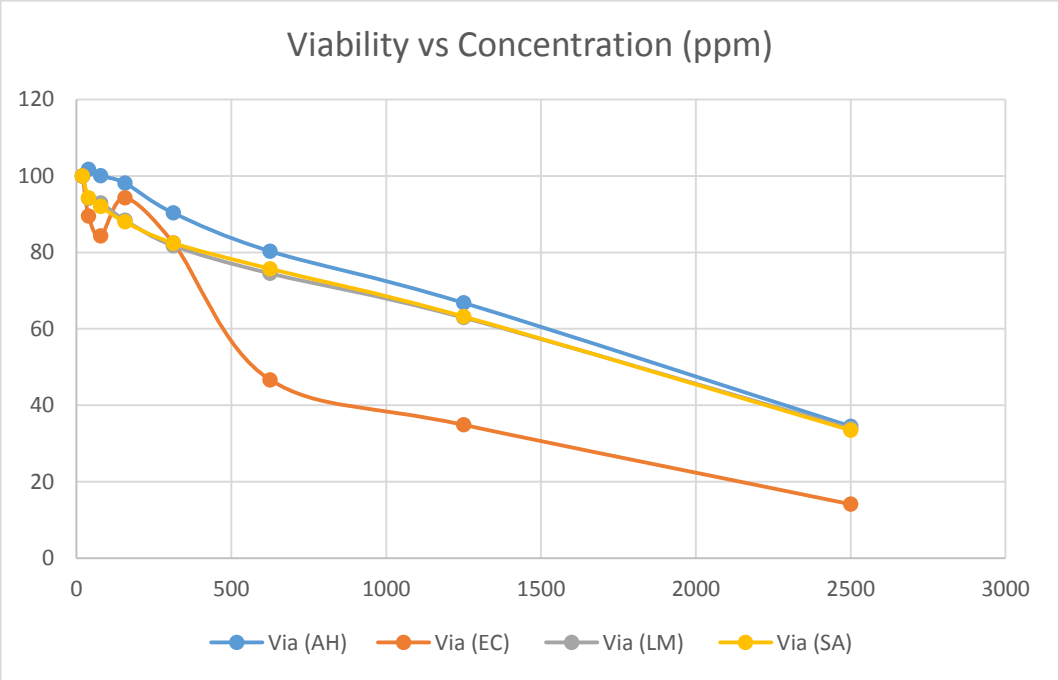

Figure 15 Graph of BMIM OSU viability vs concentration

4.1.4 1-butyl-3-methylimidazolium Hydrogen Sulphate (BMIM HSO_4)

Table 12 EC50 for BMIM HSO_4 in 96 well plate

Value	1	2	3	4	5	6	7	8	9	10	11	12	Concentration
A	0.0890	0.0903	0.1000	0.0945	0.0871	0.0862	0.0912	0.0834	0.0844	0.0797	0.0800	0.0807	37500
B	0.1216	0.1127	0.1201	0.1085	0.1909	0.1042	0.1176	0.2939	0.2791	0.1082	0.1116	0.1060	18750
C	0.1269	0.3090	0.1302	0.1120	0.1096	0.1071	0.1269	0.3257	0.3128	0.1089	0.1166	0.1274	9375
D	0.0810	0.2681	0.2855	0.1166	0.1888	0.0691	0.1340	0.3104	0.0818	0.1940	0.0878	0.0882	4687.5
E	0.9899	0.9987	1.0677	0.4826	0.4738	0.4331	1.0022	1.0894	1.0162	0.9924	1.1244	0.8539	2343.75
F	1.0676	1.0517	1.0992	0.5181	0.5009	0.3431	1.0899	1.1158	1.0981	1.0310	1.1052	1.0279	1171.875
G	1.1003	1.0742	1.0369	0.3940	0.4006	0.3939	0.9951	0.9817	0.9763	1.0004	1.0841	0.9591	585.9375
H	1.1573	1.1171	1.1028	0.5717	0.5667	0.5645	1.0608	1.0581	1.0638	0.9541	0.9360	0.8944	292.96875

Table 13 Average EC50 BMIM HSO_4 and Viability for each Microorganism (from left Aeromonas Hydrophilia, Eschericia Coli, Listeria Monocytogenes and Staphylococcus Aureus)

	Average	**Viability**	**Average**	**Viability**	**Average**	**Viability**	**Average**	**Viability**
A	0.0931	8.270165	0.0893	15.72611	0.0863	8.137745	0.0801	8.633507
B	0.1181	10.4939	0.1345	23.70075	0.2302	21.69856	0.1086	11.70048
C	0.1887	16.76241	0.1096	19.30237	0.2551	24.04876	0.1176	12.67373
D	0.2115	18.79071	0.1248	21.9919	0.1754	16.53313	0.1233	13.28784
E	1.0188	90.49805	0.4632	81.5961	1.0359	97.64665	0.9902	106.687
F	1.0728	95.30084	0.4540	79.98708	1.1013	103.8049	1.0547	113.6326
G	1.0705	95.09061	0.3962	69.79271	0.9844	92.786	1.0145	109.3051
H	1.1257	100	0.5676	100	1.0609	100	0.9282	100

Table 14 Summary viability for different concentration of BMIM HSO_4 for each microorganism

	BMIM HSO_4								**Concentration (mg/L)**
	Aeromonas Hydrophilia		**Eschericia Coli**		**Listeria Monocytogenes**		**Staphylococcus Aureus**		
	Average	**Viability**	**Average**	**Viability**	**Average**	**Viability**	**Average**	**Viability**	
A	0.0931	8.270165	0.089267	15.72611	0.086333	8.137745	0.080133	8.633507	37500
B	0.118133	10.4939	0.134533	23.70075	0.2302	21.69856	0.1086	11.70048	18750
C	0.1887	16.76241	0.109567	19.30237	0.255133	24.04876	0.117633	12.67373	9375
D	0.211533	18.79071	0.124833	21.9919	0.1754	16.53313	0.123333	13.28784	4687.5
E	1.018767	90.49805	0.463167	81.5961	1.035933	97.64665	0.990233	106.687	2343.75
F	1.072833	95.30084	0.454033	79.98708	1.101267	103.8049	1.0547	113.6326	1171.875
G	1.070467	95.09061	0.396167	69.79271	0.984367	92.786	1.014533	109.3051	585.9375
H	1.125733	100	0.567633	100	1.0609	100	0.928167	100	292.9688

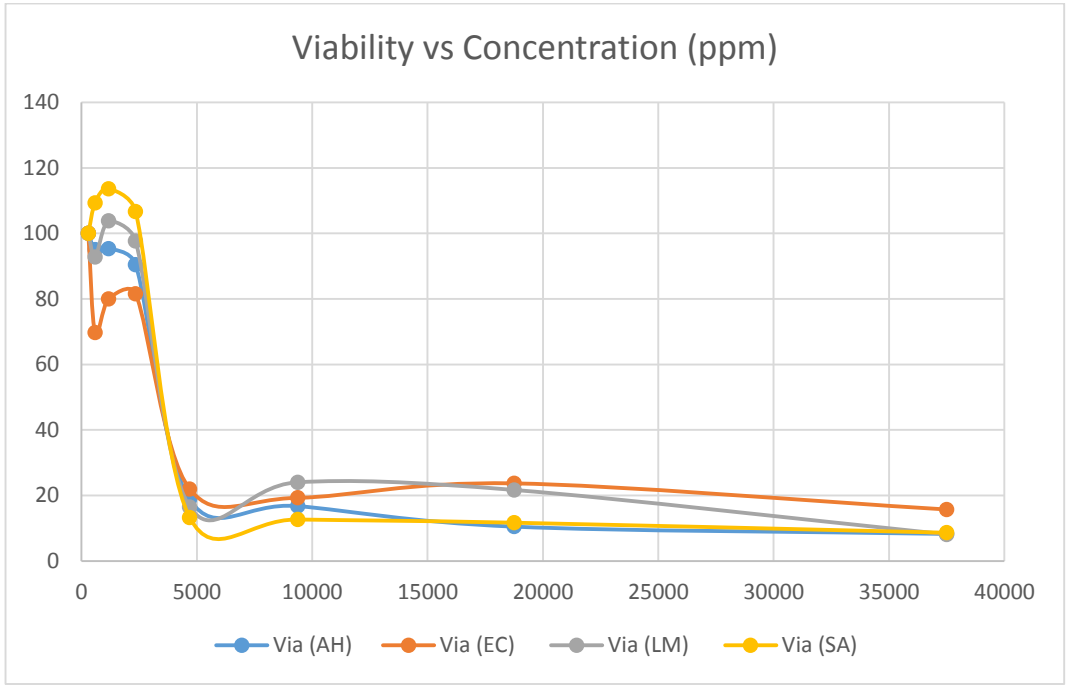

Figure 16 Graph of BMIM HSO_4 viability vs concentration

All graphs and values are summarized in table below:

Table 15 Summary of EC50 for all microorganism

	EC50 (mg/L)			
Ionic Liquid	**Escherichia Coli (Ecoli)**	**Listeria Monocytogenas**	**Aeromonas Hydrophilia**	**Staphylococcus Aereus**
MMIM DMP	error	23000	22000	25000
BMIM DMP	15500	19000	19000	16750
BMIM OSU	560	1800	1900	1800
BMIM HSO_4	3350	3400	3450	3500

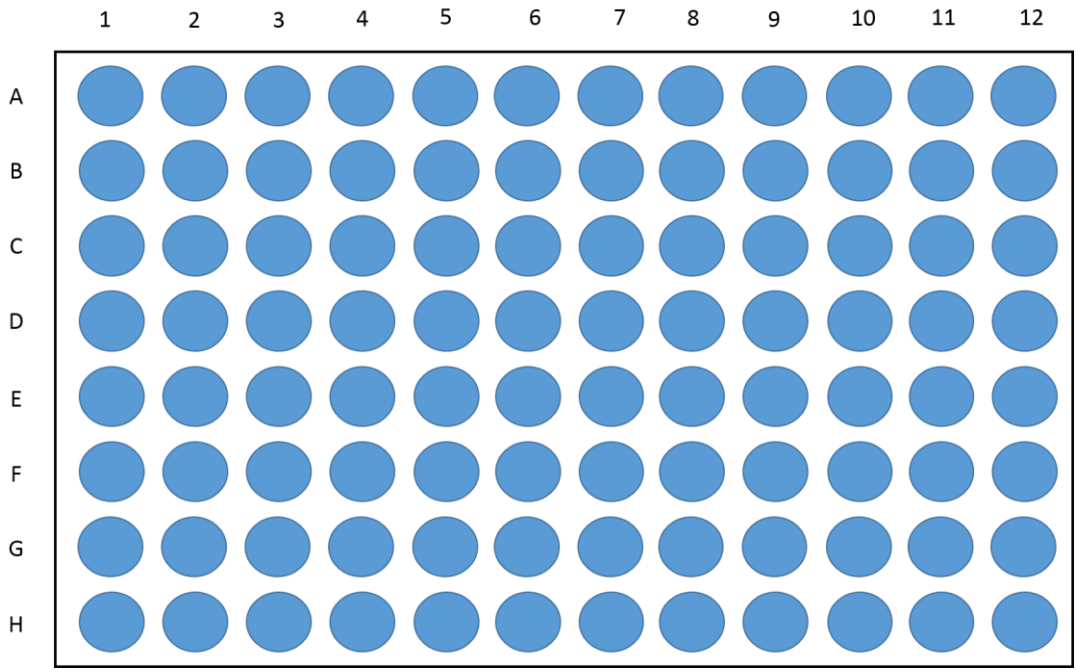

Figure 17 Division of microorganism in 96 well plate

As the plate is divided into four sections of different microorganisms, it has been labeled as:

Table 16 Division of Microorgansim in 96- well plate

Matrix	**Microorganism**
1-3	*Aeromonas Hydrophila*
4-6	*Eschericia Coli*

7-9	*Listeria Monocytogenes*
10-12	*Staphylococcus Aureus*

$$Average = \frac{Well\ for\ X1 + X2 + X3\ (depends\ on\ the\ well\ matrix)}{3}$$

From Figure 10 above, cell A is filled with chemical desired, which is the selected ionic liquid together with the bacteria according to matrix 1-12. Cell B to G is filled with serial dilution of ionic liquids, bacteria and broth. Whereas cell H is filled with bacteria and broth which will be the reference for cell A-G. The total for all wells will be 200 microLiter.

To identify the ability of the living organism whether it can maintain its potentialities, the calculations for viability towards four ionic liquids are made. For the viability of the microorganism, the last cell (cell H) is the blank solution for every plate, which contain only broth and microorganism. Thus, the calculation for viability is based on the average of concentration of cell H for every microorganism.

$$Viability = \frac{average\ of\ each\ cell}{average\ of\ last\ cell} x\ 100\%$$

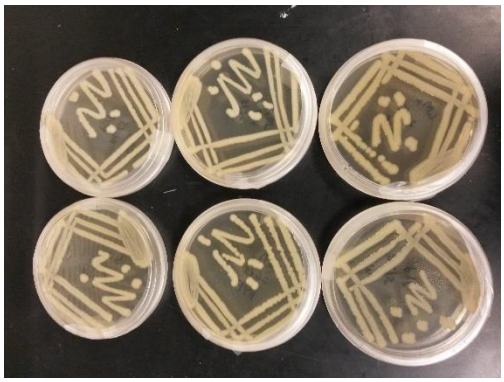

Figure 18 Microorganisms on Agar plate

Figure 19 Microorganisms on Agar (slanting) in Universal bottle

4.2 Discussion

From the graphs above, all microbes are grouped into one graph, which is then compared with four different types of ILs; MMIM DMP, BMIM DMP, BMIM OSU and BMIM HSO_4. The concentration is started off with different concentration for all ILs. The lowest number in Table 15 has the highest level of toxicity and vice versa. From the trend of result, BMIM OSU shows the most toxic value as the viability is very low compared to other ILs. This is due to the long side chain of C8. Meanwhile, MMIM DMP has the lowest toxicity level amongst the other. All graphs show good trends for the MIC test except for microorganism *Ecoli* and have achieved the targeted concentration for the inhibition of all microorganisms. The results are analyzed and discussed in general.

(i) Phosphate anion vs Sulphate anion

According to the results, we can compare both anions between sulphate and phosphate for which have more toxic level. In overall, according to Table 15, the phosphate anion shows lower toxicity level compared to sulphate anion. It turns out that both MMIM DMP and BMIM DMP are more benign than both BMIM OSU and BMIM HSO_4. Nevertheless, there are not much findings with regards to this matter. More researches are needed to support this findings.

(ii) Effect of alkyl chain

ILs are formed from the combination of anion, cation and alkyl chain. As all the researches have shown before, the level of toxicity of ILs will increase proportional to the length of alkyl chain. These results have also proved that the longer alkyl chain will have higher level of toxicity. This concept applied to both anion and cation.

Referring Table 15, BMIM OSU has the highest level of toxicity compared to all other ILs. The lowest number in Table 15 has the highest level of toxicity, which is in this experiment, BMIM OSU records 560 ppm of toxicity concentration towards *Ecoli*. We compare the toxicity level of two ILs; BMIM OSU and BMIM HSO_4. The comparison is done because of the same length of alkyl chain in cation side. Octyl- has higher C (Carbon) number compared to BMIM HSO_4; thus it gives a higher level of toxicity.

The toxicity level for anion component is also being compared. For this project, butyl- anion is compared to methyl- anion; and as known from all the studies before, the longer alkyl chain; butyl- anion will give higher toxicity level compared to methyl- anion.

(iii) Anion vs Cation

In general, it was found that the cation species is the main effector for the observed toxicity, especially if substituted with a longer alkyl side chain. The anion also contribute to toxicity, but in most cases anion effects are less drastic compared to the side chain effect. For example, let us take one microorganism to compare the level of toxicity; Staphylococcus Aereus. We compare first the difference of anion side, which are MMIM DMP and BMIM DMP. MMIM DMP shows 25 000 ppm of EC50 level, meanwhile BMIM DMP shows 16 750 ppm. The difference of these two ILs are about 8 000 ppm. In contrast, we take the anion as constant, and compare the difference of EC50 for BMIM HSO_4 and BMIM OSU which shows difference in concentration is about 2 000 ppm. For this comparison, it is proven that the cation has bigger effect towards the toxicity level of ILs.

These systematic studies are addressed to the users of ILs in different fields of application to facilitate the selection of toxicologically favorable structural elements and therefore contribute to the design of inherently safer ionic liquids.

4.3 Possible Errors

According to all four graphs, it is seen that all patterns for microorganisms *Aeromonas Hydrophila, Listeria Monocytogenes,* and *Staphylococcus Aureus* have about the same level of viability, which is about in the range of 80-100%. The only microorganism; *Eschericia Coli* has deviated from the range of curves which may due to some errors:

1. Twice preparation of test suspension for *Eschericia Coli* during test of McFarland standard. This may cause reading error in micro-plate reader during the analysis of microbes.

2. The tested microbes which already diluted within the range of McFarland standard already turbid throughout the preparation of 96-well plate. The condition in laminar flow; temperature of 37°C is very suitable for the microorganisms to grow, thus the tested microbes will be turbid throughout the experiment being conducted. From the summarized table of all ILs and microorganism, it was decided that *Ecoli* is not compatible to be done in this project. However, stipulated time is given might give a better result for *Ecoli*.

CHAPTER 5

CONCLUSION

As for the conclusion, this project is important to the society as it evaluates the level of toxicity of IL towards different types of microorganisms. Different ILs has different level of toxicity. So the research on the topic should be intensively worked out in order to identify the ecotoxicity level for different types of ILs. The project has achieve all the objectives, which evaluates the toxicity level of different concentration of MMIM DMP, BMIM DMP, BMIM OSU and BMIM HSO_4. The study has also covered the evaluation of toxicity of ILs towards different types of microorganisms namely *Aeromonas Hydrophila, Eschericia Coli, Listeria Monocytogenes,* and *Staphylococcus Aureus*. Apart from that, it is proven that the longer alkyl chain, in both anion and cation will give effect to the toxicity level of IL. Not only that, the experiment demonstrated that phosphate anion is more benign than sulphate anion.

From these conclusions, some of the data for toxicity and antimicrobial information about ILs can be provided. Therefore the design of ILs can be more "green" and prevent the pollutions from happening. Thus the cost for future clean-up can also be reduced.

RECOMMMENDATION

1. Due to the characteristics of microorganism *Ecoli*, the result for toxicity data is not fully achieved as the growth is slower than other three experimented microorganism. It is recommended that the experiment should be made several times so that the result is achieved.

2. Further research has been done and it is found that the determination of raw prediction for concentration of ILs can be done by screening. Screening is the process where the concentration can be predicted within a few ranges of concentration. By doing this, it saves time compared to preparing it in 96-well plate.

3. It is recommended that the IL which are not toxic from the experimental result can be used as an antimicrobial test as a drug delivery in pharmaceutical industry and used it as further research in cytotoxicity, which is the quality of being toxic to cells, in specifically, human cells. The non-toxic ILs can be further studied in bioprocess/biotechnology industries.

REFERENCES

1. Bernot, R. J., Brueske, M. A., Evans-White, M. A., Lambert, G.A., (2005). *Environ. Toxicol. Chem.*, 24, 87.
2. Cho, C. W., Pham, T.P.T., Jeon, Y.C., Vijayaraghavan, K., Choe, W. S., Yun, Y. S., (2007b). Toxicity of imidazolium salt with anionbromide to a phytoplankton *Selenastrum Capricornutum*: Effect of alkyl chain length. Chemosphere, 69 (2007), pp 1003-1007.
3. Danielsson, I. and Lindman, B. (1981). The definition of microemulsion. *Colloid and surfaces*, 3, 391.
4. Ferraz, R., Branco, L.C., Prudencio, C., Noronha, J.P., Petrovski, Z., (2011). "Ionic Liquids as Active Pharmaceutical Ingredients". ChemMedChem, pp 1-12.
5. FOSS, (n.d.). Analyzing for *Ecoli* Bacteria. Retrieved at 27th July, 2014 from http://www.foss.dk/search/searchnps/analyzing_for_e-coli_bacteria.html
6. Gauthier, D, LS Sobjerg, KM Jensen, AT Lindhart, M Bunge, K Finster, RL Meyer and T Skrydstrup. (2010). Environmentally benign recovery and reactivation of palladium from industrial waste by using gram-negative bacteria. ChemSusChem, 3, pp 1036-1039.
7. Ghanem, O. B., Abdul Mutalib, M.I., Leveque, J. M., Kait, C.F., El-Harbawi, M., (n.d.). *A Systematic Ecotoxicity Study of Imidazolium based Ionic Liquids towards Vibrio fischeri*, Thesis, Faculty of Chemical Engineering, Universiti Teknologi PETRONAS, Malaysia, Fundamental & Applied Sciences Department, Universiti Teknologi PETRONAS, Malaysia, Chemical Engineering Department, King Saud University, Saudi Arabia.
8. Latala, A., Stepnowski, P., Nedzi, M., Mrozik, W., (2005). Marine toxicity assessment of imidazolium ionic liquids: Acute effects on the Baltic algae *Oocystis submarina* and *Cyclotella meneghiniana*. Aqutic Toxicity, 73 (1), pp 91-98
9. Moniruzzaman, M., Goro, M., (2011). "Ionic Liquids: Future Solvents and Reagents for Pharmaceuticals". Journal of Chemical Engineering of Japan, 44 (6), pp 370-381.
10. Moniruzzaman, M., Tamura, M., Tahara, Y., Kamiya, N., Goro, M. (2010). "Ionic liquid-in-oil microemulsion as a potential carrier of sparingly soluble drug: Characterization and cytotoxicity evaluation," International Journal of Pharmaceutics, 400, pp 243-250.
11. Moniruzzaman, M., Tahara, Y., Tamura, M., Kamiya, N., Goto, M. (2010). "Ionic liquid-assisted transdermal delivery of sparingly soluble drugs". The Royal Society of Chemistry, 46, pp 1452-1454
12. Nasaruddin, M.N.H., (2010). *The Effect of cation-anion Interaction on the Toxic Properties of Ionic Liquids*, Thesis, Chemical Engineering Department, Universiti Teknologi PETRONAS, Malaysia.

13. "Preparation of Routine Media and Reagents Used in Antimicrobial Susceptibility Testing: McFarlands Standards"
14. Pretti, C., Cinzia. C., Ilaria, B., Sara, B., Gianfranca, M., Luigi, I., (2009), "Acute Toxicity of Ionic Liquids for three freshwater organism: *Pseudokirchneriella subcapitata, Daphnia magna and Danio rerio*". Ecotoxicology and Environment Safety, 72, pp 1170-1176.
15. Queen's University. (2010). Emulsions vs Microemulsions. Retrieved at 14th April, 2014 from http://chemeng.queensu.ca/courses/CHEE460/lectures/documents/Chee460_Lectures20to21_Emulsions2010.pdf
16. Reichert, W.M., (2005). The effects of cation-anion interactions on the physical and solvents properties of ionic liquids, Thesis, Department of Chemistry, University of Alabama, US
17. Rodrigues, S., Santos, P., (2010). Effect of the chain length in the structure of imidazolium ionic liquids and dimethylformamide solutions probed by Raman spectroscopy, Vibrational Spectroscopy, 54, pp 123-126
18. Siodmiak, T., Marszall, M.P., Proszowska, A., (2012). "Ionic Liquids: A New Strategy in Pharmaceutical Synthesis." Mini-Reviews in Organic Chemistry, 9 (2)
19. Vasic-Racki, D., (n.d.). The novel technologies for the use of biocatalyst and biotransformations. Faculty of Chemical Engineering and Technology, University of Zagreb.
20. Ventura, S. P. M., de Barros, R. L. F., Sintra, T., Soares, C. M. F., Lima, A. S., Coutinho, J. A. P. (2012). "Simple screening method to identify toxic/non-toxic ionic liquids: Agar diffusion test adaptation." Ecotoxicology and Environmental Safety, 83, pp 55-62
21. Wickerham, L., J. (1951). Taxonomy of yeasts, p. 11. *In* Technical bulletin no 1029. U.S. Department of Agriculture, Washington, D.C.
22. Wishart, J., Brookhaven National Laboratory. Ionic Liquids: Designer Solvents for a Cleaner World
23. Zhao, D., Liao, Y., Zhang, Z. (2007). "Toxicity of Ionic Liquids," *Clean,* 35 (1), pp 42-48

APPENDICES

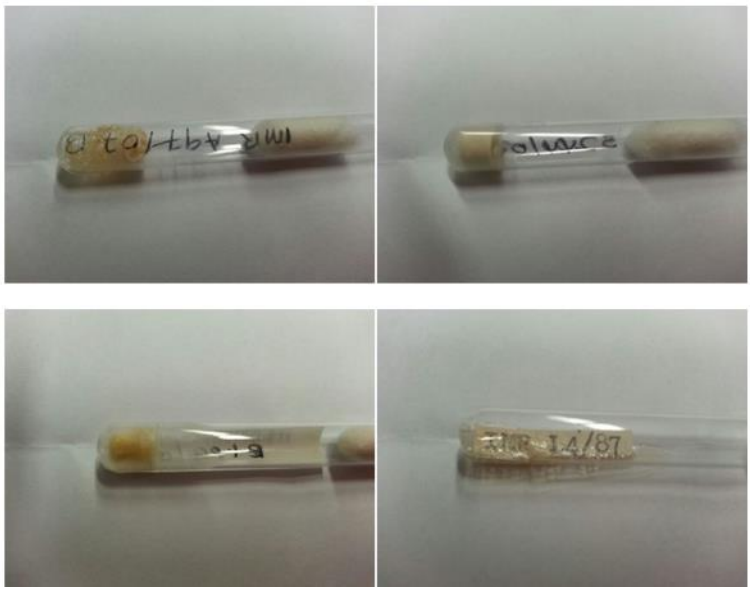

Figure 20 Appendix 1: Freeze dry of Microorganism

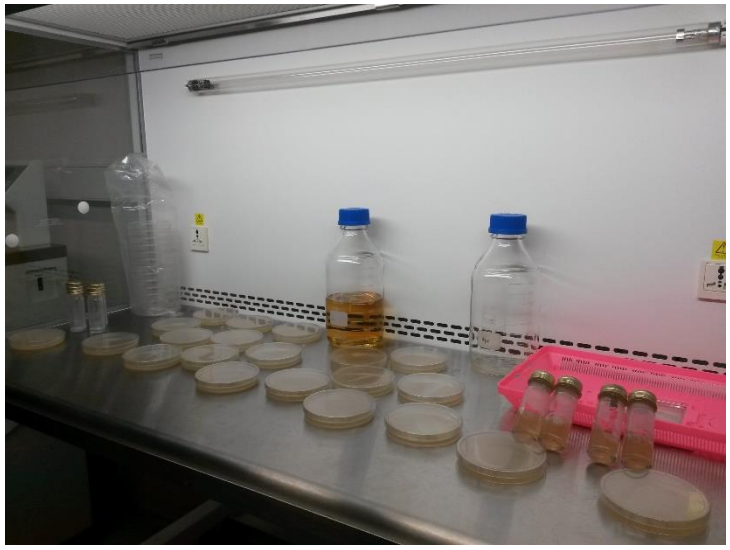

Figure 21 Appendix 2: Mueller Hinton Agar before subculture with microorganism

Table 17 Appendix 3: Solubility of substance from Remington's

Descriptive terms	Parts of solvent needed for 1 part solute
Very soluble	< 1
Freely soluble	1-10
Soluble	10-30
Sparingly soluble	30-100
Slightly soluble	100-1000
Very slightly soluble	1000-10,000
Practically insoluble or insoluble	> 10,000

Resource from http://pharmlabs.unc.edu/labs/solubility/intro.htm

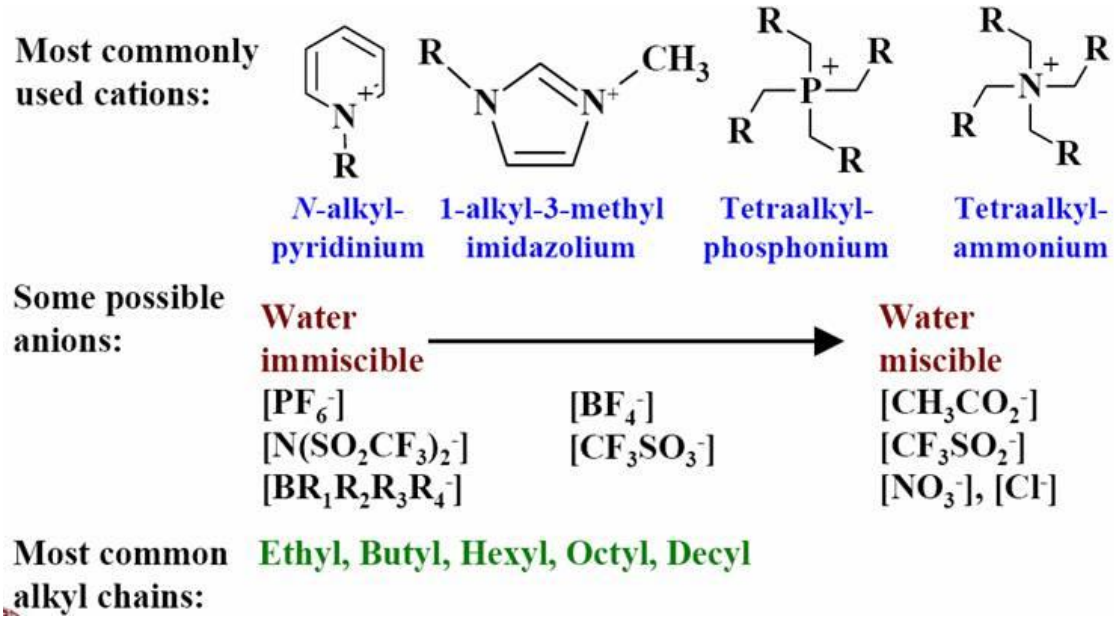

Figure 22 Appendix 4: Formulation of Ionic Liquids

Resource from
http://lem.ch.unito.it/didattica/infochimica/Liquidi%20Ionici/Composition.html

Table 18 Appendix 5: Preparation of McFarland Standard

Standard no.	Vol (ml)		No. of bacteria/ml (10^8) represented
	$BaCl_2 \cdot 2H_2O$ (1.175%)	H_2SO_4 (1%)	
0.5	0.5	99.5	1.5
1	1.0	99.0	3
2	2.0	98.0	6
3	3.0	97.0	9
4	4.0	96.0	12
5	5.0	95.0	15
6	6.0	94.0	18
7	7.0	93.0	21
8	8.0	92.0	24
9	9.0	91.0	27
10	10.0	90.0	30